June

Week 1 (30/05/16 - 03/06/16)

- MODULE 1 : Biobricks resuspension

Lyophilized DNA of BBa_K541503, BBa_K1184000 and BBa_E0040 were resuspended for further characterization.

- MODULE 2: Heat-Shock transformation of assembled biobricks

Escherichia coli DH5α strains were transformed with different vectors containing the sequences of BBa_K541503, BBa_K1184000 and BBa_E0040 biobricks.

Transformed cells were spatulated in LB Petri dishes (as a negative control) and in LB + chloramphenicol (35 μg/ml) Petri dishes to measure transformation efficiency for subsequent procedures.

Summary:

Further verification of the plasmid is still required. Process of transformation has proven effective, but it can be improved.

Week 2 (06/06/16 - 10/06/16)

In order to do the assembly for the cassette of OMVs, transformation and culturing of the proper Biobricks were performed.

- MODULE 1: Stria culturing of isolated colonies (Biobricks for characterization)

The culturing was performed for the following biobricks BBa_K1184000, BBa_K541503, and BBa_E0040.

- MODULE 2: Plasmid extraction by Mini-Prep

The plasmid extraction by Mini-Prep was performed for the following biobricks BBa_K1184000, BBa_K541503 and BBa_E0040. The BBa_J04450 extraction which was stored at -20ºC plus the ones performed in this module were loaded in an electrophoresis gel as follows:

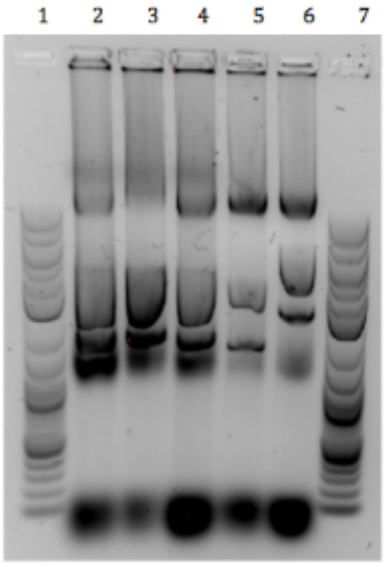

Figure 1. Electrophoresis of plasmid extraction of BBa_J04450, BBa_E0040 and BBa_K541503 in TAE 1X. Gel at 0.8% agarose. 1) Quick-Load Purple 2 – Log DNA Ladder (NEB) (5µL), 2) J04450 extraction with STE buffer using 1.5µL RNAse (8µL + 4µL LB), 3) J04450 extraction with STE buffer using 2.5µL RNAse (8µL + 4µL LB). 4) J04450 extraction with STE buffer using 3.5µL RNAse (8µL + 4µL LB) 5) E0040 extraction with STE buffer using 1.5µL RNAse (8µL + 4µL LB). 6) K541503 extraction with STE buffer using 1.5µL RNAse (8µL + 4µL LB). The gel was run at 100 V for 50 minutes and stained with GelRed 0.3X. 7) Quick-Load Purple 2 – Log DNA Ladder (NEB) (5µL).

Summary:
The results showed an unusual staining of the plasmid isoforms, which also didn't manage to show stable and uniform bands. The results indicated that too much plasmid was put inside the gel. Also, Quickload bands showed a constant curving trail. High contamination of RNA is present at the bottom of each plasmid well, independent from the quantity of RNAse used.

●MODULE 3: Enzymatic restriction

Table 1. Reactions for restriction of BBa_ K1184000

Control I (XbaI)	Control 2 (SpeI)	Digestion
32 µL nuclease free water	32 µL nuclease free water	31 µL nuclease free water
5 µL Buffer 10X (Cutsmart)	5 µL Buffer 10X (Cutsmart)	5 µL Buffer 10X (Cutsmart)
500 ug Plasmid (12 ul BBa_K1184000)	500 ug Plasmid (12 ul BBa_K1184000)	500 ug Plasmid (12 ul BBa_K1184000)
1 µL Restriction Enzyme (XbaI)	1 µL Restriction Enzyme (SpeI)	1 µL Restriction Enzyme (XbaI)
----------	----------	1 µL Restriction Enzyme (SpeI)
Total : 50 µL	Total : 50 µL	Total : 50 µL

The enzymatic restriction, was done using XbaI and SpeI enzymes in order to extract BBa_K1184000 and, at the same time, have the necessary sites for further cloning with an IPTG inducible promoter (J04500). Controls were set with each enzyme to verify their activity and then the restriction was performed with both enzymes. For each control digestion one single band was expected since the plasmid would linearize after being cut with one enzyme per control. Then for the final restriction two bands were the expected result since it was digested with two enzymes with

unique cutting sites. When cutting with a single enzyme two bands were obtained. One could be due to uncut plasmid that migrates to an unspecified size, while the second band should be the cut (linearized) plasmid.

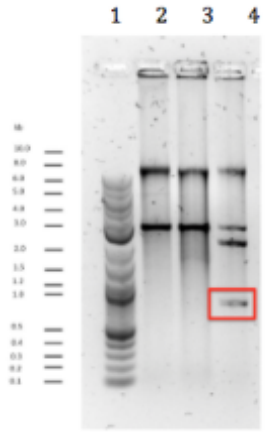

Figure 1. Electrophoresis of BBa_K1184000 extraction in TAE 0.5X. Gel at 0.8% agarose. 1) Quick-Load Purple 2 – Log DNA Ladder (NEB) (5µL), 2) Digestion using XbaI (4µL LB + 6µL), 3) Digestion using SpeI (4µL LB + 6µL) 4)Digestion using SpeI and XbaI (4µL LB + 6µL), band highlighted in red. The gel was run at 100 V for 50 minutes, and stained with GelRed 0.3X.

The marker makes it difficult to estimate the proper migration of the bands due to its circular shape, however, considering that maybe not all of the plasmid was cut with one or even with two enzymes, it can be explained that in the final lane 4 different bands can be observed that correspond to the uncut plasmid, the linearized plasmid, the band of the remaining plasmid after the biobrick was extracted and finally the band that corresponds to the biobrick.

Summary:

The extraction of plasmids from all the samples was successful since it was able to appreciate their three isoforms in each gel. Also, the quantity of RNAse that must be used in the plasmid extraction protocol in order to reduce contamination by RNA was determined.

The plasmid restriction was successful and will provide the necessary material for a future ligation. The DNA from this step was slightly degraded but after purification it should be ready to be used on the next procedure.

The assembly of the OMV's (ligation) cassette was not concluded because further analysis of the ligation in silico showed the formation of a stop codon in the scar of the ligation between the signal peptide and the coding sequence of GFP.

Week 3 (13/06/16 - 17/06/16)

In order to assemble the expression cassette of BBa_K1184000 attached to J04500, DH5alpha cells were transformed with the plasmids that contained such biobricks in order to be propagated for further plasmid documentation and digestion. Also, two new biobricks were selected in order to document their plasmids to form another expression cassette for the characterization: R0011 and K1362011.
BBa_J04500, BBa_K1184000, BBa_K11362011 and BBa_R0011 plasmid extraction.

- MODULE 1: Miniprep plasmid extraction

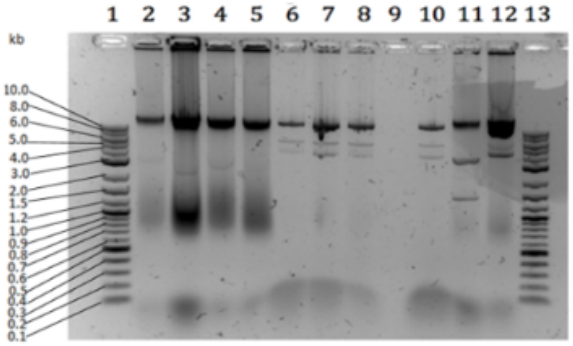

Figure 2. Electrophoresis of plasmid extraction of BBa_K1184000, BBa_J04500, BBa_K1362011 and BBa_R0011 in TBE 0.5X. Gel at 0.8% agarose. **1)** Quick-Load Purple 2 – Log DNA Ladder diluted 1:3 in TE buffer (NEB) (3µL + 4µL LB), **2)** R011 extraction using 3µL RNAse (5µL + 4µL LB), **3)** R011 extraction using 3µL RNAse (5µL + 4µL LB), **4)** R011 extraction using 3µL RNAse (5µL + 4µL LB), **5)** R011 extraction using 3µL RNAse (5µL + 4µL LB), **6)** K1362011 extraction using 3µL RNAse (5µL + 4µL LB), **7)** K1362011 extraction using 3µL RNAse (5µL + 4µL LB), **8)** K1362011 extraction using 3µL RNAse (5µL + 4µL LB), **9)** This Lane was skipped, **10)** K1362011 extraction using 3µL RNAse (5µL + 4µL LB), **11)** J04500 extraction using 3µL RNAse (5µL + 4µL LB), **12)** K1184000 extraction using 3µL RNAse (5µL + 4µL LB).**13)** Quick-Load Purple 2 – Log DNA Ladder diluted 1:3 in MB grade water (NEB) (3µL + 4µL LB). The gel was run at 110 V for 50 minutes and stained with GelRed 0.3X.

BBa_J04500 (located in the eleventh well): Three bands (two of them corresponding to plasmid isoforms)can be appreciated, this meaning a clearly contamination with genomic DNA.

BBa_K1184000 (located in the twelfth well): Three bands can be observed, indicating a high genomic DNA contamination due to the thickness of the band.

BBa_K11362011 (from the sixth to the tenth well): Two plasmid isoforms (the supercoiled and relaxed circle ones) can be appreciated, plus a clear contamination with genomic DNA in all four wells.

BBa_R0011(from the second to the fifth well): Two plasmid isoforms can be seen (mostly in well two, three and four), but we can also observe that there is genomic DNA contamination.

●MODULE 3: Plasmid restriction (BBa_J04500)

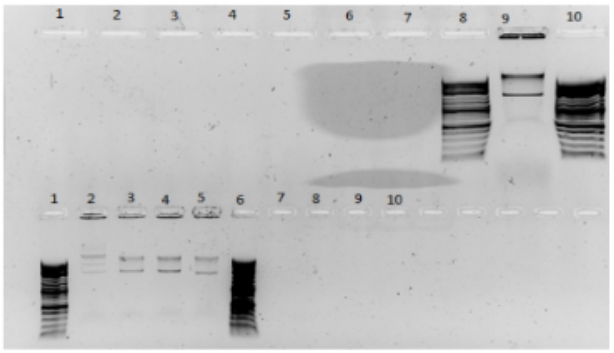

Figure 3. Electrophoresis of plasmid digestion of BBa_J04500 in TBE 0.5X Gel at 0.8% of agarose. In the big wells: **8)** Quick-Load Purple 2 – Log DNA Ladder diluted 1:3 in MB grade water (NEB) (5µL + 4µL LB), **9)** J04450 plasmid digestion with EcoRI & SpeI restriction enzymes (30µL + 6µL LB), **10)**Quick-Load Purple 2 – Log DNA Ladder diluted 1:3 in TE buffer (NEB) (5µL + 4µL LB). In the small wells: **1)** Quick-Load Purple 2 – Log DNA Ladder diluted 1:3 in MB grade water (NEB) (5µL + 4µL LB), **2)** J04450 plasmid digestion with XbaI (5µL + 4µL LB) , **3)** J04450 plasmid digestion with SpeI (5µL + 4µL LB), **4)** J04450 plasmid digestion with EcoRI & SpeI (5µL + 4µL LB), **5)** J04450 plasmid digestion with EcoRI (5µL + 4µL LB), **6)** Quick-Load Purple 2 – Log DNA Ladder diluted 1:3 in TE buffer (NEB) (5µL + 4µL LB). This gel was run at 100V for 25 minutes stained with Gel Red 0.3X.

In making plasmid digestions one must be careful at using the restriction enzymes, so they don't present star activity and start cutting in unespecific places. For the BBa_J04500 biobrick the enzymes used were EcoRI & SpeI. EcoRI being located in the 3118 bp of the plasmid and SpeI being in the 1071 bp of the plasmid. The bands obtained in the big wells (ninth well) the digestion made for the BBa_J04450 biobrick can be seen, the gel was run for 25 minutes so the results cannot be very specific, because the bands didn't migrated enough including the ones in the ladder (wells nine and twelve). In order to see if the digestion was well made another gel must be run with only one line of wells and with the corresponding conditions (50min, 100V), in this gel we are expecting to see a band with 243 bp and a band with 2,047 bp.

Summary

The extraction of plasmids from all the samples was successful since it was able to appreciate their two isoforms in each gel. Also, the quantity of RNAse that must be used in the plasmid extraction protocol in order to reduce contamination by RNA was determined. There's still a possibility that we may look for another RNAse that works better. It was also evident in the electrophoresis procedure, that TAE and TBE carried their function better out when used at 0.5X, reason why it will be the preferent way of usage in future electrophoresis protocols in order to obtain precise, reliable results and therefore expect high quality results in further procedures.

Week 4 (20/06/16 - 24/06/16)

In order to assemble the cassettes responsible for the expression of KillerRed (J04500+K1184000) and the cassette responsible for the expression of the Lambda-Lysozyme (R0011+K1362011) previously isolated biobrick plasmids were digested using the standard assembly protocol, the bands of interest were purified from the agarose gel using PureLink® Quick Gel Extraction Kit and then they were ligated using T4 Ligase. Once we obtained the cassettes, they were propagated in order to be able to isolate cassette plasmids.

- MODULE 1: Miniprep plasmid extraction of BBa_R0011, BBa_J04500 and BBa_K11362011. In order to increase efficicency and quality of the protocol, the total time of incubation with both the lysozyme and STE buffer was reduced to 4 minutes, and the amount of RNAse added per eppendorf microtube was increased to 4μL.

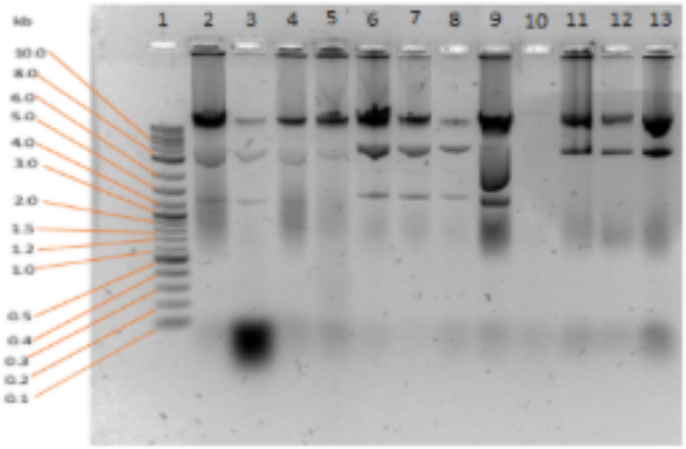

Figure 4. Electrophoresis of plasmid extraction of BBa_R0011, BBa_J04500 and BBa_K11362011 in a 0.8% agarose gel run in a 0.5 X TBE buffer at 100 V for 50 minutes stained with GelRed 0.3 X. **1)** Quick-Load Purple 2-Log DNA ladder diluted 1:3 in MB grade water (NEB) (3µL + 4 µL LB), **2)** R0011 plasmid extraction, **3)** R0011 plasmid extraction, **4)** R0011 plasmid extraction, **5)** R0011 plasmid extraction, **6) ,7),8), 9)** J04500 plasmid extraction, **11), 12), 13)** K1362011 plasmid extraction.

Plasmid extraction of BBa_R0011, BBa_J04500 and BBa_K136201. In the second, third and fourth well corresponding to R0011 biobrick, it is possible to visualize two isoforms, while in the fifth well, only one isoform can be seen. In this case, it is the relaxed circular one. In the sixth, seventh, eighth and ninth well, a plasmid isolation was performed due to the presence of the two previously mentioned isoforms. In the eleventh, twelfth and thirteenth well, only one isoform can only be seen. All wells present genomic DNA contamination as well as RNA presence.

●MODULE 2: Restriction

Table 1. Restriction conditions and result that defines the ligation strategy.

Part	E 1	E 2	Buffer	Result
J04500	EcoRI	SpeI	2.1	The promoter sequence is extracted.
K1184000	EcoRI	XbaI		The plasmid is opened and has sites to directly insert the promoter before the part.

The reaction conditions for the restriction digestion of R0011 and K1362011 are described on **Table 3** and **Table 4**

The reaction conditions for the restriction digestion of R0011 and K1362011 are described on Table 2 and Table 3.

Table 2. Reaction conditions for the restriction digestion of R0011

Element	Control P	S+P (with RNA)	S+P(without RNA)
H_2O MB	32μL	31μL	31μL
Buffer (2.1)	5μL	5μL	5μL
Plasmid	12μL	12μL	12μL
PstI	1μL	1μL	1μL
SpeI	------	1μL	1μL

Table 3. Reaction conditions for the restriction digestion of K1362011

Element	Control X	Control P	X+P
H_2O MB	32μL	31μL	31μL
Buffer (3.1)	5μL	5μL	5μL
Plasmid	12μL	12μL	12μL
XbaI	1μL	------	1μL
PstI	------	1μL	1μL

●MODULE 3:

KillerRed cassette containing BBa_J04500 coupled to BBa_K1184000 was succesfully transformed in DH5***α*** cells, culture was made in a LB + CAM medium.
Two plates were inoculated since there were two ligation reactions and both were tested. Both tubes contained the ligation expected and it was able to be introduced into competent cells. From one

plate, eight isolated colonies were chosen and stria cultured in a new plate in order to obtain pure strains.

Summary:
The assembly and transformation of the cassette containing J04500 + K1184000 was achieved, unfortunately due to certain situations at the lab, the documentation of the cassette that contains R0011+K1362011 could not be achieved, but they were transformed. Further analysis of the cassettes such as PCR or restriction assays are still recommended.

Week 5 (27/06/16 - 01/07/16)

Cassette assembly of BBa_K11362011, and BBa_R0011 (an IPTG inducible promoter: lacI regulated, lambda pL hybrid); a RBS-λ-lysozyme was assembled. Cassette was transformed into Escherichia coli BL21 competent cells and then, documented so that a further characterization of this biobrick could be performed.

This cassette assembly is achieved by digesting plasmids previously isolated (R0011 and K1362011), purifying bands of interest so that the resulted DNA parts (a linearized plasmid and a RBS-coding sequence with available sticky ends) could be ligated for a subsequent transformation in Escherichia coli BL21 competent cells.
Documentation analysis has to be carried out before performing an induction and solubility analysis.

- MODULE 1: Restriction

Table 1. Restriction conditions and result that defines the ligation strategy and cassette verification.

Part	E 1	E 2	Buffer	Result
R0011	SpeI	PstI	2.1	There is a linearized plasmid and has sites to directly insert K1362011 after the promoter.
K1362011	XbaI	PstI		There is a DNA fragment of approximately 550 bp ready to be ligated to the plasmid backbone that contains R0011
J04500+K1184000	EcoRI-HF	PstI		The expected result is a DNA fragment of approximately 600 bp.

- MODULE 2: Purification

For BBa_R0011 which acts as the plasmid backbone of the cassette, the expected bands were of 18 bp and 2116 bp. The band is acceptable for purification from gel, as it is not faint. The purified band was used for the ligation of the cassette assembly of BBa_R0011 and BBa_K1362011, which was transformed using BL21.

- MODULE 3: Ligation

In Petri dishes it is clear that the eight colonies that were taken from the culture of the Transformation.

Table 2 Transformation of three ligations (a R0011-K1362011 ligation executed in June 20th, 2016, a R0011-K1362011 ligation executed in June 21th, 2016 and a J04500-K1184000 ligation) were spatulated in different plaques:

Ligation	LB+ competent cells	LB + transformed cells	LB with AMP + competent cells	LB with AMP + transformed cells	LB with CAM+competent cells /LB with CAM + transformed cells
R0011-K1362011 (june 20 th)	performed	performed	performed	performed	---------
R0011-K1362011 (june 21th)	--------	----------	---------	performed	----------
J04500-K1184000	performed	performed	----------	----------	performed

Summary:
The assembly, transformation and documentation of the cassette containing R0011 + K1362011 was accomplished. This was due to an efficient digestion of both biobricks with their respective restriction enzymes. Once they were ligated, it was possible to verify the presence of the cassette assembly by means of carrying out a gel electrophoresis by using the first restriction enzyme (EcoRI) and the last enzyme (PstI) of the standard assembly method. Last but not least, the R0011+K1362011 ligation was verified by transforming E. coli BL21 competent cells in LB medium with AMP.